

Gemin5 promotes IRES interaction and translation control through its C-terminal region

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ABSTRACT

Gene expression control largely depends on ribonucleoprotein complexes regulating mRNA translation. Initiation of translation in mRNAs that overcome cap-dependent translation inhibition is often driven by internal ribosome entry site (IRES) elements, whose activity is regulated by multifunctional RNA-binding factors. Here we show that Gemin5 interacts preferentially with a specific domain of a viral IRES consisting of a hairpin flanked by A/U/C-rich sequences. RNA-binding assays using purified proteins revealed that Gemin5–IRES interaction depends on the C-terminal region of the protein. Consistent with this novel finding, the C-terminal region of Gemin5, but not the N-terminal region, impaired translation. Furthermore, RNA selective 2′hydroxyl acylation analysed by primer extension (SHAPE) reactivity demonstrated that addition of purified Gemin5 to IRES mRNA induced the specific protection of residues around the hairpin of the IRES element. We further demonstrate that Gemin5 out-competed SHAPE reactivity variations induced by the IRES-binding factor PTB, leading to a local conformational change in the IRES structure. Together, our data unveil the inhibitory mechanism of Gemin5 on IRES-mediated translation.

INTRODUCTION

RNA-binding proteins play a crucial role in gene expression control in all organisms. In eukaryotic cells, a large variety of ribonucleoprotein complexes affect the processing, transport, localization, translation and decay of mRNAs (1–5). Thus, RNA-binding proteins are responsible for the establishment and regulation of RNA–protein networks that determine the target mRNA fate. The untranslated regions (UTR) of mRNAs play a key role in many of these processes, serving as platforms for the

assembly of macromolecular complexes particularly those controlling translation initiation (6).

Initiation of translation in eukaryotic mRNAs involves a group of specialized proteins, known as initiation factors (eIFs) that recruit the small ribosome subunit to the m⁷GpppN residue (or cap) located at the 5′-end of most mRNAs (7). However, strong cellular stresses (including viral infection, cell death, nutrient deprivation, heat-shock, oxidative stress, DNA damage, among others) repress cap-dependent initiation of translation. Thereby, cap-independent mechanisms have been exploited by mRNAs translated under these situations (8–10). Initiation of translation in some viral RNAs, exemplified by picornaviruses, is governed by internal ribosome entry site (IRES) elements (11). In addition to a subset of eIFs, IRES-dependent translation of viral and cellular mRNAs depends on host factors termed IRES transacting factors (ITAFs) (12–16). ITAFs are RNA-binding proteins that regulate RNA lifespan as part of macromolecular complexes operating either in the nucleus or in the cytoplasm of the cell (17,18). However, the mode of action used by distinct ITAFs to regulate the activity of different IRES elements remains unclear.

Using riboproteomic approaches, we identified Gemin5 as one of the proteins that has the capacity to bind viral IRES elements (19). The interaction of Gemin5 with the IRES elements of foot-and-mouth disease virus (FMDV) and hepatitis C virus (HCV) was direct, as shown by immunoprecipitation of photo-crosslinked RNA–protein complexes (20). Functional analysis addressing the role of Gemin5 showed that this factor down-regulated IRES activity and adversely affected cap-dependent initiation, suggesting that this protein acts as a general down-regulator of translation (20). Subsequently, Gemin5 was reported to photocrosslink to 7-methylguanosine (21), and more recently, Gemin5 was included in the mRNA interactome list (22). Together, these findings revealed a multifunctional role for the Gemin5 protein, acting on translation control beyond its previously described role in small nuclear ribonucleoproteins (snRNP) biogenesis (23,24). Because a significant fraction of Gemin5 is

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The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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present in the cell cytoplasm not associated with the survival of motor neurons (SMN) complex (25,26), it could be envisioned that Gemin5 performs unanticipated functions in gene expression control. However, how this protein recognizes distinct RNA targets and whether this function resides in different domains of the protein remains elusive.

Gemin5 is proteolysed in FMDV-infected cells; this event depends on the expression of L protease and takes place at similar times as cleavage of other RNA-binding proteins such as poly(A)-binding protein (PABP) and polypyrimidine-binding protein (PTB) (27). Notably, Gemin3 cleavage has also been reported to occur during enterovirus infection (28), suggesting that inactivation of the SMN complex may be a general feature of picornavirus infections.

Here we uncover the mechanism by which Gemin5 interacts with the IRES element and represses translation. We have found that the C-terminal region of Gemin5 is responsible for the IRES recognition interacting with the most 3' end domain of the IRES element, upstream of the initiator codon. Furthermore, we show that the C-terminal region of Gemin5 acts as a translation repressor; this property is achieved by the capacity of Gemin5 to interfere with PTB, one of the RNA-binding proteins interacting with this IRES region (19,29,30). In agreement with these results, selective 2' hydroxyl acylation analysed by primer extension (SHAPE) RNA reactivity assays demonstrated that the binding of Gemin5 to the IRES element specifically induced the protection of residues within domain 5 and, interestingly, out-competed with the effects of PTB on SHAPE reactivity.

MATERIALS AND METHODS

Constructs

Plasmids expressing different domains of FMDV IRES element were described (29,31). Domain 5 mutants were amplified with the sense primers d5II-wt, d5II-base, or d5II-stem (Supplementary Table S1), in conjunction with SP6 primer. PCR products were digested with EcoRI and BamHI and ligated into pGEM3 vector, similarly treated. Construct 5-rest, restoring the proximal stem of the hairpin, was performed by PCR mutagenesis using primers sd5IIbase and asd5IIrest (Supplementary Table S1) with pGEM-d5 wt as template, followed by a second PCR with asd5II, and inserted into the EcoRI/XhoI sites of pGEM. Construct 5-U/A was performed by overlapping PCR using the pair of primers sd5IIwt, asd5IIU/A and sd5IIU/A, asd5II (Supplementary Table S1) with pGEM-d5II template. The PCR product was inserted into the pGEM vector by EcoRI/BamHI digestion. Constructs 5-sm and 5-scr were generated using primers sd5IIsm and asd5II, or sd5IIscr and asd5II, respectively. PCR products were inserted into the EcoRI site of pGEM.

Mutagenesis of the IRES element present in the pBIC construct (expressing the bicistronic RNA CAT—FMDV IRES—luciferase) to insert the sm and scr substitutions was performed using the Quickchange mutagenesis procedure (Agilent Technologies) with the pair of primers

sId5-sm, asId5-sm, and sId5-scr, asId5-scr, respectively (Supplementary Table S1). All plasmids were sequenced to ensure correctness.

The construct pRSETBG5-13WD, encoding His-Xpress tagged G5-13WD protein, was generated from an intermediate construct (pRSETBG5.1) that contained nt 1-2273 from pcDNA3.1D-Gemin5 V5/His (20) flanked by BamHI restriction sites, followed by insertion of the Sall-EcoRI 1811 nt fragment from pcDNA3.1D-Gemin5 V5/His into pRSETBG5.1. The construct expressing His-Xpress tagged G5-Cter protein was generated using primers sG5.1EcoRI and asG5EcoRI, and pcDNA3.1D-Gemin5 V5/His template. This product (nt 3859 to 4527) was inserted into the EcoRI site of pRSETB. The construct pRSETBG5, expressing His-Xpress Gemin5 protein, was generated by insertion of the EcoRI fragment from pRSETBG5-Cter into pRSETBG5-13WD. The construct pGEMG5-Cter was generated by amplifying the entire Gemin5-coding region from pcDNA3.1D-Gemin5 V5/His using primers sG5.2Eco and asG5Sall, and inserted into EcoRI/Sall sites of pGEM. The nt sequence of all constructs was verified by sequencing (Macrogen).

RNA synthesis

Transcription was performed for 1 h at 37°C using 1000–3000 U of T7 RNA polymerase with 10–15 µg of linearized DNA, 40 mM Tris-HCl, 50 mM DTT, 0.5 mM rNTPs, as described (32). When needed, transcripts were uniformly labeled using α -³²P-CTP (500 Ci/mmol) with 10 U of T7 RNA polymerase and 1 µg of linearized plasmid (33). RNA was extracted with phenol-chloroform, ethanol precipitated and resuspended in 10 mM Tris, pH 8, 1 mM EDTA (TE) to a concentration of 0.04 pmol/µl. RNA integrity was examined in 6% acrylamide, 7 M urea denaturing gel electrophoresis.

Domain 5 RNAs were generated from pGEM-d5IIwt, 5-base, 5-rest, 5-U/A, 5-stem, 5-sm and 5-scr constructs linearized with *Xho*I. Monocistronic RNA was prepared from pIC plasmid (34) linearized with *Bbu*II prior to generate an RNA encompassing the IRES element linked to firefly luciferase. Bicistronic RNAs [chloramphenicol acetyl transferase (CAT)—IRES—luciferase (LUC)] were produced as described (35). To obtain RNAs expressing G5-13WD or G5-Cter, plasmids pRSETBG513WD and pGEMG5Cter were linearized with *Xba*I or *Sall*, respectively.

Expression and purification of proteins

Escherichia coli BL21 bacteria transformed with plasmids pRSETBG5, G5-13WD or G5-Cter were grown in autoinduction medium (36) overnight at 37°C. Bacterial cell lysates were prepared in binding buffer (20 mM NaH₂PO₄, 500 mM NaCl, 20 mM Imidazol) using a French press, and cell debris was eliminated by centrifugation at 16000g 30 min at 4°C twice. The lysate was loaded in His-GraviTrap columns (HealthCare), and the recombinant protein was eluted using Imidazol 500 mM. Recombinant 6x-His tagged PTB was similarly purified using Ni-agarose columns. Proteins were dialysed

against phosphate buffer pH 6.8, 1 mM DTT, and stored at -20°C in 50% glycerol.

RNA–Gemin5 binding assays

Protein G-agarose/Xpress/Gemin5 complexes were assembled by incubating protein G-agarose beads (30 μl) (SIGMA), pre-cleared four times with PBS, with the anti-Xpress antibody (200 ng) (Invitrogen) overnight at 4°C in a rotating wheel. Unbound antibody was removed by washing four times in cold BBH buffer (10 mM HEPES; pH 7.4, 100 mM NaCl, 2.5 mM MgCl_2 , 0.01% NP-40), prior to resuspending the beads in 400 μl of BBH buffer. Then, 100 μl aliquots of antibody beads were incubated with, or without, Gemin5 or G5-13WD or G5-Cter (0.7 pmol) in the presence of 100-fold excess of BSA during 4 h at 4°C in a rotating wheel. Unbound proteins were removed by washing four times with cold BBH buffer. ^{32}P -labelled domain 5 RNA (120 fmol) was added to each protein-beads complex and incubated in cold BBH buffer in the presence of cytoplasmic RNA (200-fold excess), during 2 h at 4°C in a rotating wheel. Unbound RNA was removed by washing four times with cold BBH buffer. Bound RNAs were extracted with phenol–chloroform, ethanol precipitated, and fractionated by 6% acrylamide, 7 M urea denaturing gel electrophoresis, and visualized by autoradiography.

RNA–protein photocrosslinking and immunodetection

BHK-21 cytoplasmic cell extract was prepared as described (37). Uniformly radiolabelled probes (0.04 pmol, $\sim 5 \times 10^5$ cpm) were incubated with S10 cell extracts (40 μg total protein) or purified proteins (50–200 ng) and UV-irradiated in the presence of 1000-fold molar excess of total cytoplasmic RNA (20). Following extensive RNase treatment, samples were subjected to 8% SDS-PAGE, and ^{32}P -labelled proteins were visualized by autoradiography.

For immunoprecipitation of RNA–protein complexes, pre-cleared protein A-sepharose beads (SIGMA) were incubated with Gemin5 antibody (Bethyl) overnight at 4°C in a rotating wheel. Unbound antibody was removed, and the beads were incubated with the RNA–protein UV-crosslinking products (20). Unbound proteins were removed by washing four times with radioimmunoprecipitation assay (RIPA) buffer and once with PBS. Bound proteins were fractionated by 8% SDS-PAGE.

Gemin5 was immunodetected by western blot as described (27) using anti-Gemin5 antibody (Novus). Secondary antibodies (Thermo Scientific) were used according to the manufacturer instructions.

In vitro translation

G5-13WD and G5-Cter transcripts (0.03–0.45 pmols) synthesized *in vitro* were translated in 70% rabbit reticulocyte lysate (RRL) (Promega) supplemented with ^{35}S -methionine (10 μCi) 15 min prior to addition of the bicistronic RNA (200 ng) bearing the FMDV IRES, as described (27).

IRES activity assays

Relative IRES activity was quantified as the expression of luciferase normalized to that of CAT from bicistronic mRNAs as described (34) in transfected BHK-21 monolayers. Experiments were performed on triplicate wells, and each experiment was repeated at least three times.

SHAPE RNA probing in the presence and absence of IRES-binding factors

RNA–protein complexes were assembled in folding buffer (100 mM HEPES pH 8.0, 6 mM MgCl_2 , 100 mM NaCl) using 0.17 μM RNA (38) in the presence of increasing amounts of HIS-tagged PTB (100, 500 or 900 nM), Gemin5 or the C-terminal region of Gemin5 (15, 75, or 750 nM) during 10 min at room temperature. Then, RNA alone or preincubated with the protein of interest was treated with *N*-methylisatoic anhydride (NMIA) (39).

For RNA–protein complexes assembled using two proteins, the lowest concentration of each protein rendering a protection pattern was used to add increasing concentrations of the other factor. Briefly, a constant concentration of PTB (500 nM) was incubated with RNA in the presence of Gemin5 (75 or 300 nM). Conversely, a constant concentration of Gemin5 (75 nM) was incubated with RNA in the presence of PTB (500 or 900 nM).

For primer extension, equal amounts of NMIA-treated and untreated RNAs (10 μl) were incubated with 0.5 μl of the appropriate antisense 5' end ^{32}P -labelled primer (5' CT ACGAAGCAACAGTG, 5' CCCGGGTGTGGGTACC, 5' GGAATGGGATCCTCGAGCTCAGGGTC, 5' GGC CTTTCTTTATGTTTTTGCG). Primer extension was conducted in a final volume of 15 μl containing reverse transcriptase (RT) buffer (50 mM Tris HCl pH 8.3, 3 mM MgCl_2 , 75 mM KCl, 8 mM DTT) and 1 mM of each dNTP. The mix was heated at 52°C for 1 min, prior to addition of 100 U of Superscript III RT (Invitrogen) and incubation at 52°C for 30 min. cDNA products were fractionated in 6% acrylamide, 7 M urea gels, in parallel to a sequence obtained with the same primer (38).

Data from two independent assays were used to calculate the mean SHAPE reactivity. For this, the intensity of each RT-stop band was normalized to the total intensity of the gel lane made relative to the corresponding full-length product intensity (set to 100%). Then, the background values of the untreated RNA (NMIA–) were subtracted from the respective RT-stop intensity yielded by the treated RNA (NMIA+). To obtain SHAPE differences in RNA–protein complexes, the SHAPE reactivity values obtained in the samples incubated with free RNA were subtracted from the reactivity values obtained in the presence of protein, run in parallel.

RESULTS

Gemin5 protein specifically binds to domain 5 of the IRES

We have shown that Gemin5 binds to the FMDV IRES (20). However, neither the target region within the IRES element that provides the binding site for Gemin5 nor the region of the protein responsible for the IRES interaction was known. To identify the binding site of Gemin5 within

the IRES element, we generated transcripts corresponding to separate IRES domains, termed 1-2, 3, 4-5, 5, 5-H and 5-ss (Figure 1A). These transcripts were used as probes to perform photocrosslinking assays, by incubating ³²P-labelled RNAs with soluble cell extracts, a method that reveals factors covalently linked to the probe (Figure 1B). Polypeptides p220, p120 and p80 interacting with the FMDV IRES have been previously identified as eIF4G, eIF3b/c and eIF4B, respectively (29). The 170 kDa protein that corresponds to Gemin5 (20) interacted with domain 5. Dissection of domain 5 into the hairpin (5-H) and the single stranded region (5-ss) indicated that 5-H contained the preferential binding site of p170, while the probe 5-ss barely interacted with it (Figure 1C). In contrast, the entire domain 5 was required to detect efficient p80 crosslinking.

To verify whether the UV-crosslinked polypeptides corresponded to Gemin5, we conducted immunoprecipitation assays with anti-Gemin5 antibody (Figure 1D). Notably, the p170 product was detected with probes 5 and 5-H. In both cases, failure of p80 immunoprecipitation confirmed the assay specificity. Furthermore, no immunoprecipitation products were detected with probes 1-2 and 5-ss. We conclude that Gemin5 specifically binds to the hairpin of domain 5 within the IRES element.

Systematic mutagenesis of domain 5 reveals that Gemin5 binding allows RNA sequence flexibility

Domain 5 consists of a hairpin of nine base pairs, followed by a single-stranded region that incorporates a polypyrimidine-rich sequence (Figure 1E). Covariation analysis of sequences found in multiple isolates of FMDV indicated that the stem structure (nt 419–440) is conserved. In contrast, the most 3' end region of domain 5 is variable although the pyrimidine tract (nt 438–447) is conserved (32). Despite this sequence variability, this region partly mirrors the Sm site of snRNAs, consisting of a 5' AUUU UUG-3' sequence upstream of a hairpin (23).

Because the 5-H probe consisting uniquely of the hairpin, but not the 5-ss RNA, was capable of interacting with p170 in a UV-crosslink assay (Figure 1C and D), we determined the role of the stem in Gemin5–IRES interaction by destabilizing its RNA structure. A mutant RNA disrupting the three apical base pairs (5-stem), but retaining the proximal part of the hairpin (Figure 1E), was not affected in p170 binding, although a diminished p80 interaction was detected, in agreement with other mutants altering the apical loop of domain 5 (29). A mutant disrupting three base pairs of the proximal region of the hairpin (5-base) and a second-site mutant aimed to restore RNA structure (5-rest) did not disable p170 interaction, while p80 binding was abolished in the 5-base mutant.

We next examined the involvement of residues located at the 5'-end of the hairpin in Gemin5 interaction using mutants 5-sm and 5-scr (Figure 1F). Notably, interaction of p170 with these probes was slightly enhanced relative to the wild type (wt) RNA, while p80 binding was abolished. Nonetheless, the RNA 5-sm bearing the AAUUUUUGA sequence was similarly efficient in p170 binding capacity than the RNA 5-scr, bearing the ACCACGUGA

sequence. To further dissect the residues responsible for Gemin5 interaction, we generated the mutant 5-U/A (Figure 1F), bearing five U residues within the polypyrimidine tract substituted to A. Compared with RNA wt, this probe also showed a moderate increase in its capacity to bind p170, suggesting that the U residues placed at the 3'-end of domain 5 were not a determinant for the interaction with Gemin5. Together, these results led us to conclude that interaction of Gemin5 with the IRES element allows RNA sequence flexibility, in agreement with the sequence variability of FMDV RNA (32). Notwithstanding, a short hairpin in the context of domain 5 sequence, absent in other IRES regions, is sufficient to provide a landing pad for this protein.

In support of the biological relevance of Gemin5 interaction with the IRES element, bicistronic constructs bearing the sm or scr substitution exhibited a decreased IRES activity in transfected BHK-21 cells (Supplementary Figure S1), with the peculiarity that the IRES element harbouring the sm substitution was 3-fold more defective than the one carrying the scr substitution.

Gemin5 directly interacts with the IRES element through its C-terminal region

Gemin5 is a 170 kDa protein with 13 WD repeats at the N-terminal region (40). Earlier work has reported that the fifth WD repeat is responsible for the interaction with the Sm site of snRNAs (23). As shown above, U-rich sequences were not specifically required for Gemin5 interaction with the IRES element. The lack of similarity between the IRES binding site and the Sm site found in snRNAs suggested that the region of the protein involved in IRES interaction could be different.

Thus, to determine the Gemin5 region involved in the interaction with the IRES element, we generated His-tagged versions of the protein spanning amino acids 1–1508 (full-length Gemin5), 1–1288 (G5-13WD) and the C-terminal moiety containing amino acids 1287 to 1508 (G5-Cter). These proteins also contain an Xpress epitope at the amino-terminal end (Figure 2A). ³²P-labelled domain 5 was incubated with His-tagged purified proteins (either Gemin5, G5-13WD or G5-Cter) immobilized on protein G-agarose beads coupled to anti-Xpress antibody (Figure 2B). Bound RNAs were isolated and analysed by denaturing polyacrylamide gel electrophoresis (Figure 2B). The mobility of the isolated RNAs was similar to the free probe (lane 1), loaded as Mw marker. The sample containing RNA incubated with protein G-agarose/antiXpress beads, but no protein (lane 2), served as background signal (42% relative to the signal observed in lane 3 with Gemin5 set at 100%). Notably, the RNA-binding capacity of the C-terminal region (G5-Cter) (lane 5, 108%) was similar to that of the full-length protein (Gemin5) (lane 3), and higher than that of G5-13WD truncated protein (lane 4, 39%). This result indicated that the C-terminal region spanning amino acids 1287 to 1508 of Gemin5 is sufficient to interact with the FMDV IRES.

To further validate this conclusion, we conducted a UV-crosslink assay using increasing amounts of purified

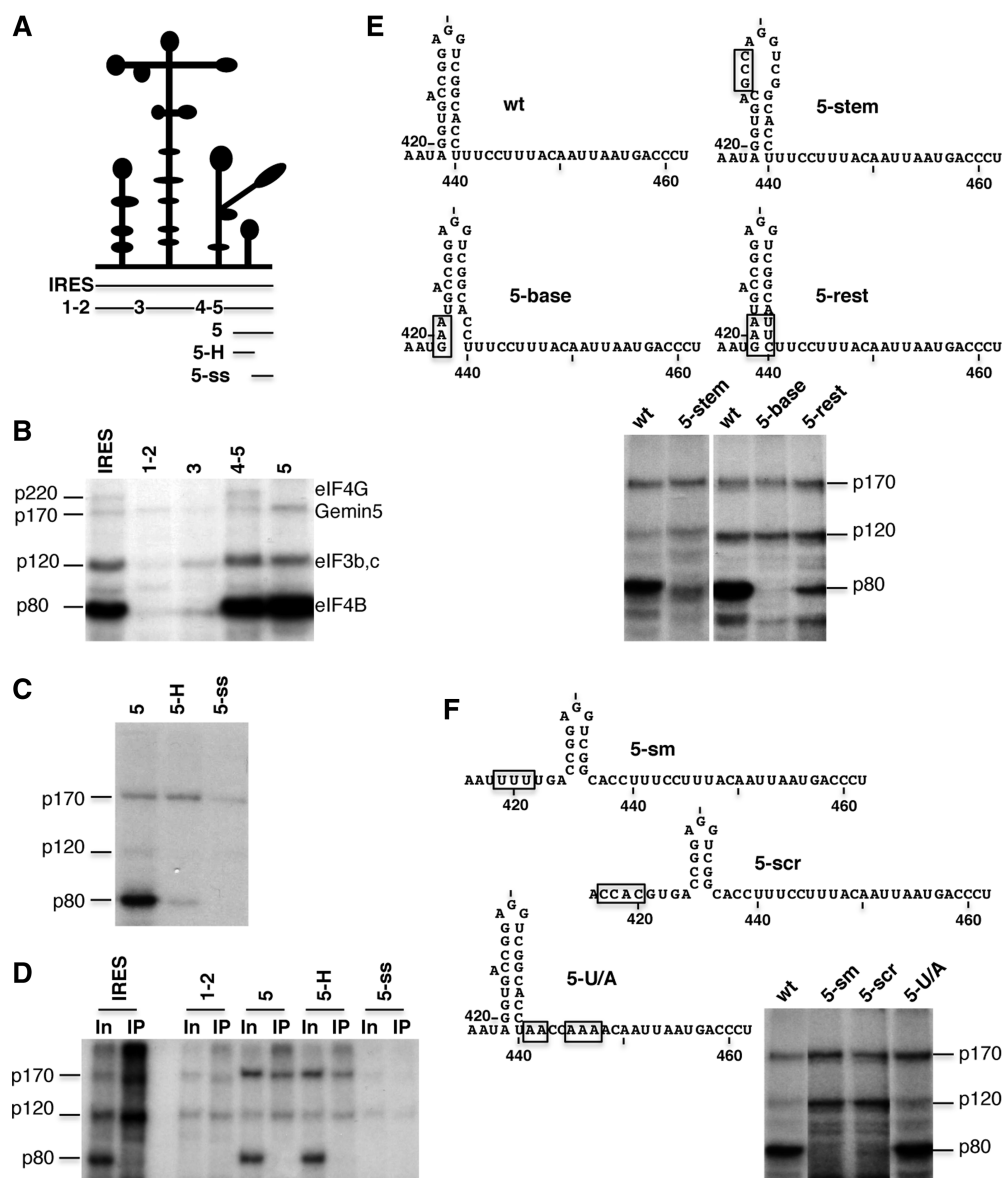


Figure 1. Gemin5 binds directly to domain 5 of the FMDV IRES. (A) Secondary structure of the FMDV IRES with indication of structural domains. Thin lines at the bottom depict the approximate location of transcripts (IRES, 1-2, 3, 4-5, 5, 5-H and 5-ss) used in this work. (B) UV-crosslinking assay of BHK-21 S10 cell extracts with radiolabelled IRES, or domain 1-2, 3, 4-5 and 5 RNAs. Arrows indicate the position of p220, p170, p120 and p80 photo-crosslinked products. (C) UV-crosslinking assay of radiolabelled domain 5, domain 5 hairpin (5-H) and the single-stranded region of domain (5-ss). (D) Immunoprecipitation of Gemin5 from BHK-21 cell extracts photo-crosslinked to radiolabelled transcripts IRES, 1-2, 5, 5-H and 5-ss. Samples were fractionated on 8% SDS-PAGE. In all cases the input (In) corresponds to 5% of the immunoprecipitation (IP) sample. (E) Effect of destabilization of the hairpin of domain 5 in protein photocrosslinking. Secondary structure of wild type (wt) domain 5 (corresponding to probe 5 in Figures 1B–D); substitutions designed to destabilize the hairpin in mutants 5-stem or 5-base, and to restore the RNA structure in mutant 5-rest are marked with a rectangle (top panel). Autoradiograph of a UV-crosslinking assay carried out with the indicated 32 P-labelled mutant RNAs and BHK-21 S10 extracts, fractionated on SDS-PAGE 8% (bottom panel). Thin lines point to p170, p120 and p80. (F) Effect of the primary sequence composition within the 5' and 3' region of the hairpin in protein photocrosslinking. Substitutions designed to modify the primary sequence of the 5' (mutants 5-sm, 5-scr) and 3' sequence (5-U/A) of domain 5 (top panel) are marked with a rectangle. UV-crosslinking assay of the indicated 32 P-labelled mutant RNAs and BHK-21 S10 extracts (bottom panel).

G5-Cter and 32 P-labelled domain 5 (Figure 2C). A UV-crosslinking product of about 40 kDa, with the same mobility as the protein analysed by western blot using anti-Gemin5 antibody, was detected. No crosslinking products were observed using similar concentrations of purified G5-13WD protein (Supplementary Figure S2), irrespectively of the addition of S10 BHK-21 cell extracts to the UV-crosslink assay potentially

containing auxiliary factors. Therefore, we conclude that the C-terminal region of Gemin5 directly interacts with domain 5 of the IRES element.

The C-terminal region of Gemin5 down-regulates translation

Gemin5 downregulates translation in tissue cultured cells as well as in cell free systems (20). To determine whether

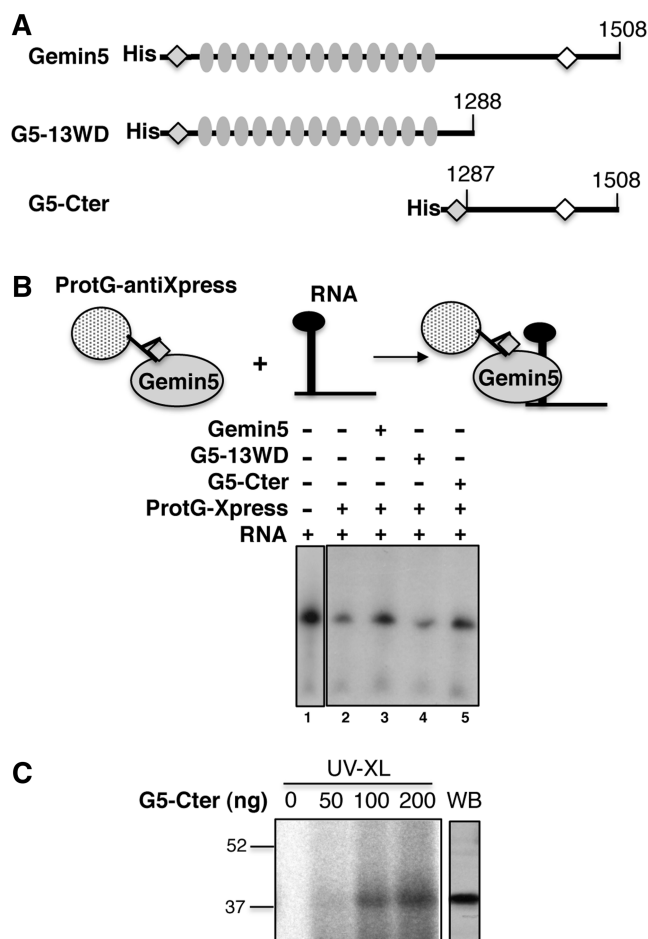


Figure 2. Identification of the Gemin5 region involved in IRES interaction. (A) Diagram of the His-tagged Gemin5 proteins used in this study. Numbers indicate the amino acids encompassed by Gemin5 (full-length protein), G5-13WD (N-terminal region) or G5-Cter (C-terminal region). The Xpress epitope at the N-terminal end present in all proteins and the epitope recognized by anti-Gemin5 antibody are depicted by a grey or white diamonds, respectively; grey ovals depict the 13 WD repeats. (B). Schematic representation of the RNA-binding assay (top). Dotted circles depict agarose beads bound to anti-Xpress antibody; Xpress-tagged Gemin5 is depicted by grey ovals with a grey diamond, radiolabelled RNA is depicted in black. Autoradiograph of a denaturing 6% acrylamide gel, 7M Urea loaded with RNAs isolated from protein G-Xpress antibody beads coupled to the indicated proteins (bottom). (C) UV-crosslinking (UV-XL) assay conducted with increasing amounts (0 to 200 ng) of purified His-tagged G5-Cter and radiolabelled domain 5. The mobility of the same protein detected by western blot (WB) using anti-Gemin5 is shown on the right.

or not the IRES-binding capacity of the C-terminal region of Gemin5 correlated with the ability to control translation, we generated RNAs expressing truncated forms of the protein from the T7 promoter, one corresponding to the amino-terminal region of the protein (G5-13WD), and the other corresponding to the C-terminal region (G5-Cter). These RNAs were used in *in vitro* translation assays to measure their capacity to interfere bicistronic RNA translation (Figure 3A and B). Of interest, the 13WD polypeptide did not affect the IRES-dependent translation efficiency of luciferase, while only a very modest reduction (10%) of CAT synthesis was observed at the

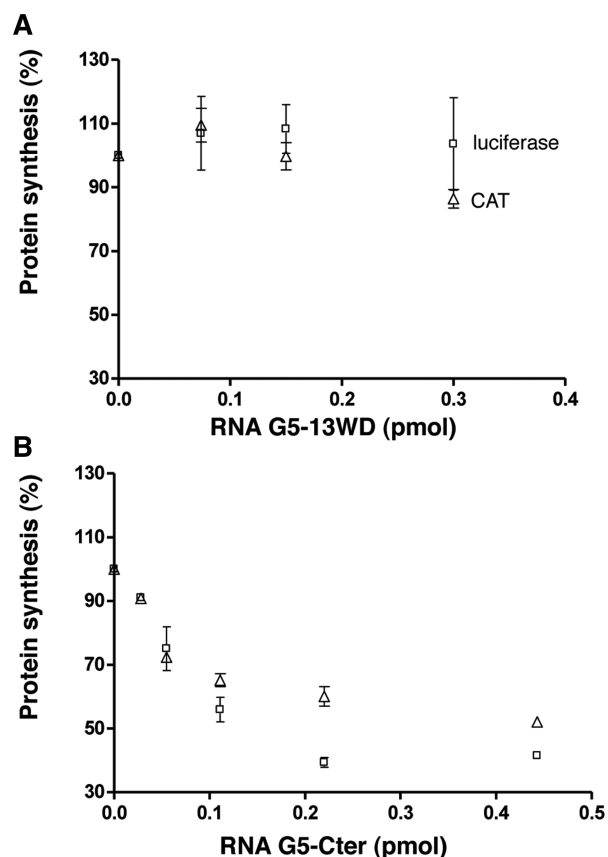


Figure 3. The C-terminal region of Gemin5 represses translation. (A) Increasing amounts of RNA expressing G5-13WD were added to RRL prior to addition of a bicistronic RNA (200 ng) bearing the FMDV IRES in the intercistronic region. 35 S-labelled proteins were resolved in 12% SDS-PAGE, and the intensity of 35 S-labelled luciferase (squares) (IRES-dependent translation) and CAT (triangles) (5'-end dependent translation) proteins was measured in a densitometer. (B) Increasing amounts of an RNA expressing the C-terminal region of Gemin5 (G5-Cter) were added to RRL prior to addition of a bicistronic RNA. Values correspond to the mean (\pm SD) of three assays.

highest concentration of G5-13WD RNA (Figure 3A). In contrast, expression of the G5-Cter protein induced a dose-dependent decrease of both, IRES-dependent and 5'-end-dependent translation efficiency (Figure 3B). Thus, we conclude that the C-terminal region of Gemin5 is responsible for the translation repression activity of this RNA-binding protein.

Gemin5 induces the protection of residues belonging to domain 5 in IRES-protein complexes assembled *in vitro*

To establish a relationship between RNA-protein interaction and repression of IRES activity, we analysed the changes in local nucleotide flexibility by selective 2'-hydroxyl analysed by primer extension (SHAPE), a methodology that allows the study of long RNA molecules, either free (41) or assembled in RNA-protein complexes (42). To this end, RNA encompassing the entire IRES element was incubated with increasing amounts of purified Gemin5 protein prior to NMIA treatment. Reverse-transcriptase extension analysis (32) allowed the measurement of SHAPE reactivity of the IRES region on

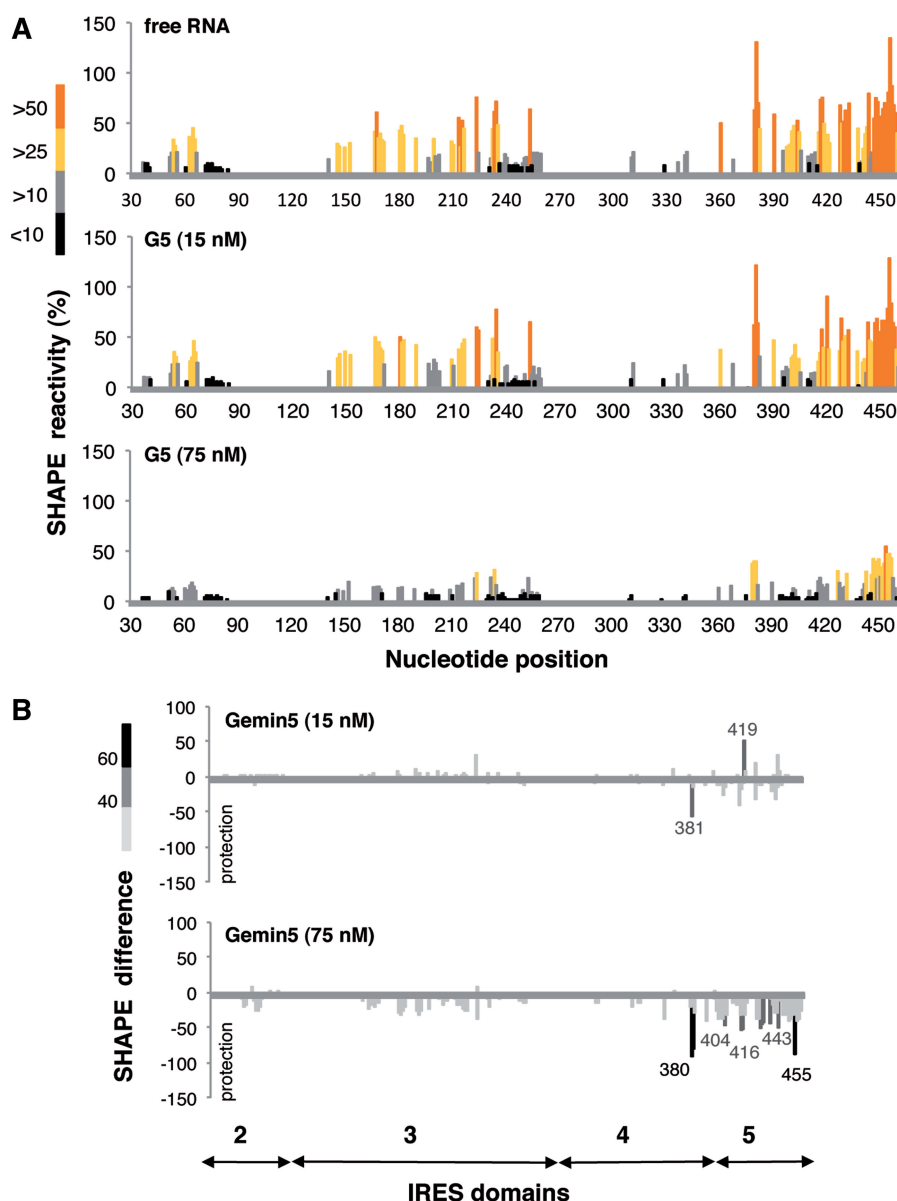


Figure 4. Impact of Gemin5 on IRES SHAPE reactivity. (A) RNA SHAPE reactivity as a function of nucleotide position for the free RNA treated with NMIA is depicted at the top panel. SHAPE reactivity values observed on addition of purified Gemin5 (15, 75 nM) are shown (top to bottom panels). Bars are coloured according to reactivity. Numbers depict nucleotides showing larger differences in SHAPE reactivity. (B) SHAPE difference plots of the IRES RNA incubated with Gemin5 relative to free RNA. Arrows below the bottom panel indicate the border of IRES domains 2, 3, 4 and 5.

RNAs incubated, or not, with Gemin5. SHAPE reactivity reflects the intensity of the NMIA-treated RNA primer extension products, normalized to the corresponding untreated RNA, in the presence or absence of different concentrations (0, 15, 75 nM) of Gemin5 (Figure 4A).

The SHAPE reactivity values observed with free RNA were used to compute differences in SHAPE reactivity observed upon addition of Gemin5 (Figure 4B). Interestingly, specific RNA residues become protected from NMIA attack. The protections observed include RNA regions that report RNA–protein interactions, or changes in RNA conformation induced by the RNA–protein interaction. Upon addition of Gemin5, SHAPE reactivity differences revealed a dose-dependent induced protection of

nucleotides spanning domain 5 and the most 3′-end of domain 4 (nt 380–458). Of interest, 75 nM Gemin5 was sufficient to induce the protection of residues belonging to domain 5. Residues belonging to domains 2 and 3 showed no gross changes in SHAPE differences (Figure 4B). These results revealed that Gemin5 specifically interacts with residues belonging to domain 5, in agreement with the results shown in Figure 1D.

Similar assays carried out with the C-terminal moiety of Gemin5 indicated that this region of the protein is sufficient to induce the protection of the sequences that lie nearest to the 3′-end of the IRES (Supplementary Figure S3), reinforcing the conclusions from data in Figure 2B and C. Although the protection was less intense than that

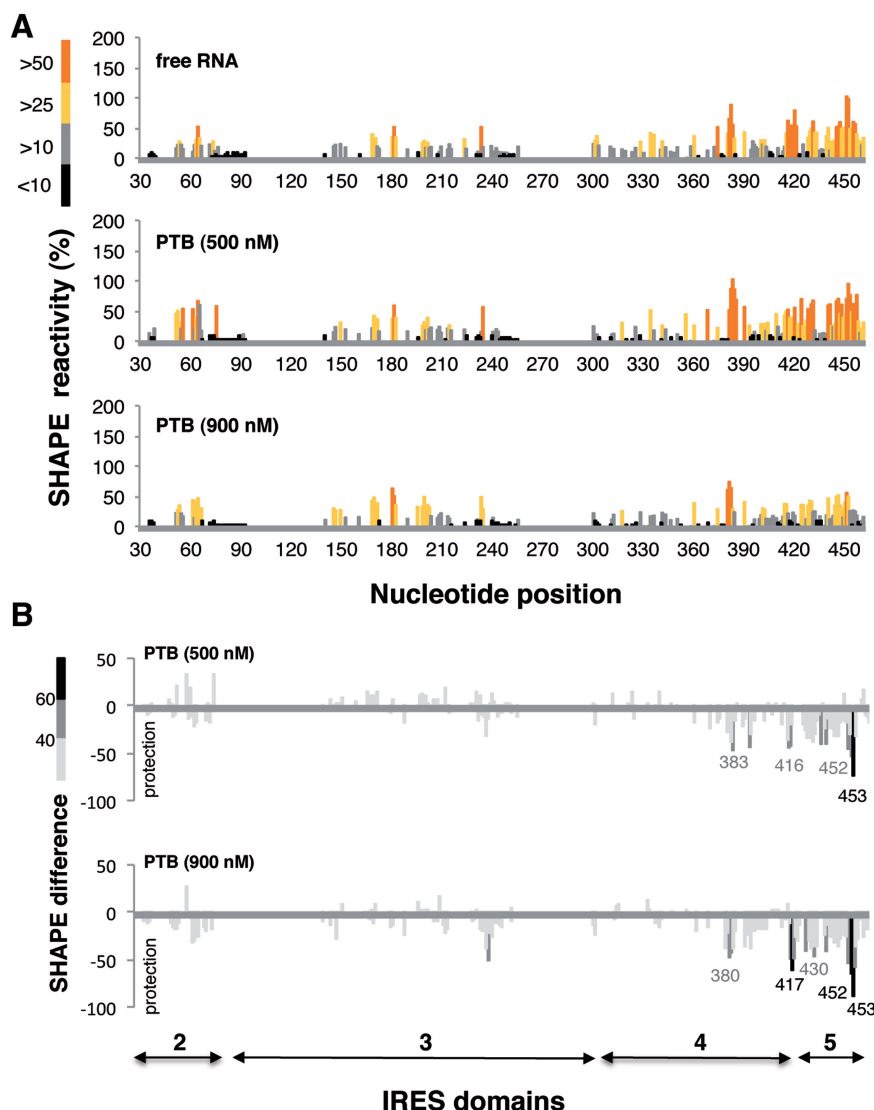


Figure 5. Dose-dependent effect of PTB on IRES RNA structure monitored by SHAPE. (A) RNA SHAPE reactivity as a function of nucleotide position for the free RNA is depicted at the top panel. SHAPE reactivity values observed upon addition of purified PTB (500, 900 nM) are shown (top to bottom panels). (B). SHAPE difference plots of the IRES RNA incubated with PTB relative to free RNA. Numbers depict nucleotides showing larger differences in SHAPE reactivity. Arrows below the bottom panel indicate the border of IRES domains 2, 3, 4 and 5.

observed with the full-length protein, the protected residues map to the same IRES region. An increase in SHAPE reactivity noticed at nt 451 on addition of G5-Cter was not observed with the entire protein.

The RNA region recognized by Gemin5 in UV-crosslink assays (Figure 1B and C) overlaps with the binding site of PTB within the FMDV IRES (29,43). Thereby, the results shown in Figure 4A prompted us to verify whether or not the interaction of Gemin5 with the IRES element could affect the binding of PTB. For this, SHAPE assays were conducted with increasing concentrations (500 or 900 nM) of purified PTB individually (Figure 5A). On addition of PTB, SHAPE differences relative to the free RNA indicated that residues preferentially protected from NMIA attack included positions 417–453 (Figure 5B).

In summary, and relative to the SHAPE reactivity observed with free RNA (Figure 6), incubation of RNA

with individual proteins led to a decreased SHAPE reactivity at specific positions within domains 4 and 5. Notably nt 380–382, 416–418, 427–432 and 440–458 were protected from NMIA attack upon addition of Gemin5. These protections partially overlapped with those observed on incubation of the IRES element with PTB (Figure 6). Together, the results derived from SHAPE local nucleotide dynamics allow us to conclude that residues with decreased flexibility on addition of Gemin5 partially overlap with those induced by the IRES-binding protein PTB.

Gemin5 out-competes PTB from its RNA-binding site in IRES–protein complexes

Next, to evaluate a potential interference between PTB and Gemin5 in IRES recognition, we conducted SHAPE reactivity assays of IRES–protein complexes using

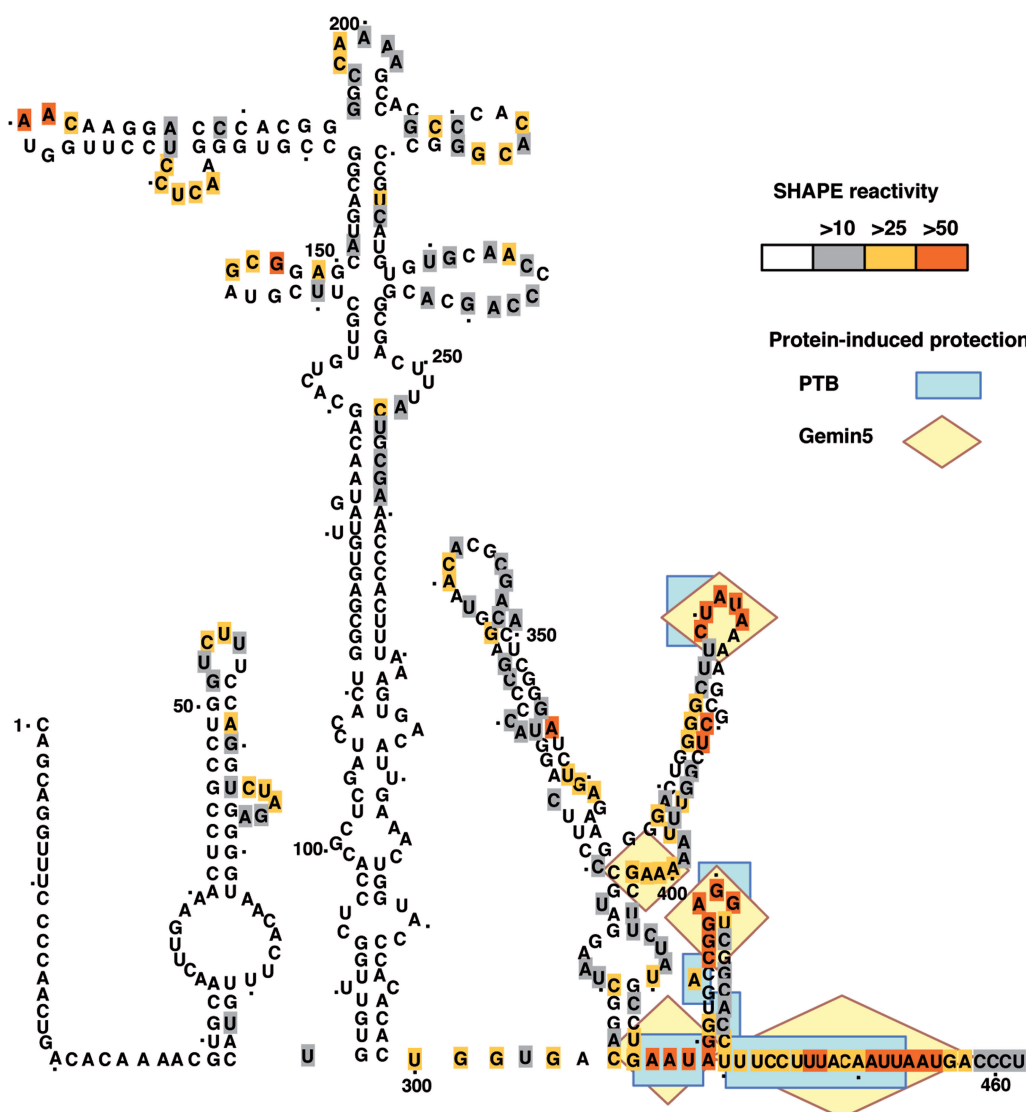


Figure 6. Summary of SHAPE reactivity of the IRES element. Nucleotide SHAPE reactivity observed in the free RNA is represented by a coloured scale (>10, grey; >25, yellow; >50, orange). The nucleotides protected from RNA attack in SHAPE reactivity assays upon addition of Gemin5, or PTB, are depicted by yellow diamonds and blue rectangles, respectively.

combinations of two factors within a concentration range previously determined to detect protein-induced RNA protection (Figures 4 and 5). Supplementary Figure S4 shows a representative example of a SHAPE reactivity assay using increasing amounts of Gemin5 in the presence of a constant amount of PTB (lanes 3–7), and conversely, a fixed amount of Gemin5 in the presence of increasing concentrations of PTB (lanes 8–12). As expected, the NMIA-treated RNA (+) (lane 2) in the absence of proteins showed a marked reactivity compared with the untreated RNA (–) (lane 1). This result denoted the residues with high SHAPE reactivity using naked RNA (Figure 6).

Quantitative analysis of SHAPE reactivity obtained with each RNA–protein complex assembly condition was performed to determine the differences in SHAPE reactivity induced by incubation of the RNA with proteins, relative to

the reactivity of free RNA. SHAPE differences derived from two independent experiments performed with PTB and Gemin5 revealed that low amounts of Gemin5 (75 nM) interfered with the interaction between PTB (900 nM) and domain 5 (Figure 7A). For simplicity, only the SHAPE difference profile along IRES nt 300 to 462 is shown, as no changes in residues belonging to domains 2 and 3 were observed. Addition of higher concentrations of Gemin5 led to a similar SHAPE difference pattern (Supplementary Figure S5). On the other hand, a conformational change in RNA structure that exposed the most 3' end residues of the polypyrimidine tract (CUUUACAA) was observed in both cases (Figure 7B). We conclude that Gemin5 exerts its inhibitory activity by interfering with the recognition of the translation activator PTB. Thus, beyond the well-described SMN complex, Gemin5 forms part of a ribonucleoprotein complex that controls IRES activity.

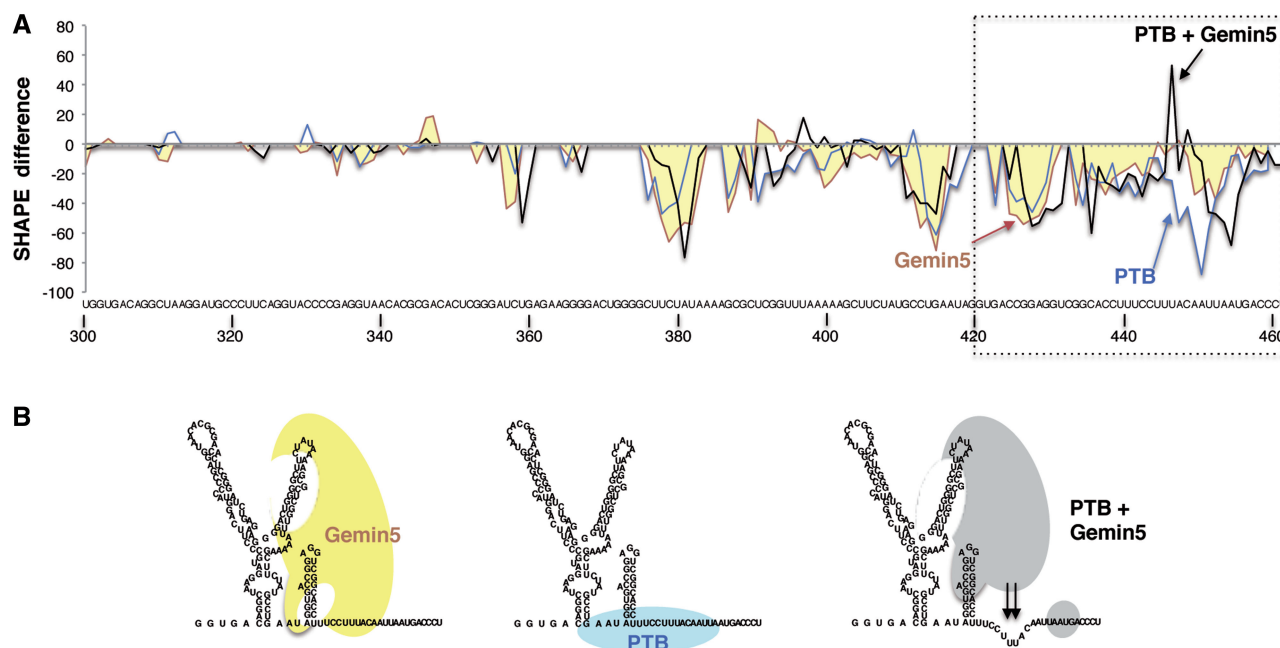


Figure 7. Modification of the difference in SHAPE reactivity induced upon addition of Gemin5 and PTB. (A) SHAPE difference profiles of the IRES incubated with Gemin5 (75 nM), PTB (900 nM), alone or combined, relative to free RNA. The RNA region where most of the rearrangements are observed upon addition of the proteins is highlighted by a dotted rectangle. (B) Schematic representation of the Gemin5 (yellow) and PTB (blue) individual binding sites and the conformational reorganization of domain 5 (highlighted with arrows) induced by the combined addition of both proteins (grey).

DISCUSSION

Gemin5 was identified as the protein that delivers the SMN complex to the Sm site of snRNAs (23), as an IRES-binding factor (19) and as a translation down-regulator (20). Here we report three main findings relevant to the activity of Gemin5 as a translation regulator. First, we show that the C-terminal part of Gemin5 possess RNA-binding capacity being responsible for the interaction with the 3' end domain of a picornavirus IRES. Second, we show that the C-terminal region of the protein contributes to down-regulate translation efficiency, and third, we provide evidence that this may occur through partial competition with PTB for the RNA sequence encompassing domain 5 of the IRES element.

The RNA-binding capacity of this region of Gemin5 is a novel finding. In snRNAs the Gemin5 recognition motif has been described as a U-rich sequence, usually preceding a short stem-loop, although some differences exist (44). In contrast, U-rich sequences are not specifically required in the IRES element for Gemin5 binding. Photocrosslinking assays aided by immunoprecipitation led to the identification of domain 5 as the binding site of Gemin5 within the IRES element. Additionally, an extensive mutational analysis indicated that binding of Gemin5 allows a large RNA sequence flexibility. Nonetheless, a common feature of snRNAs and the IRES-binding site found here is the presence of a short hairpin flanked by sequences rich in A/U/C residues. This feature has been shown in high throughput sequencing analysis of novel snRNAs precursors bound to Gemin5 (26). Reinforcing this conclusion, substitution of five Us by As in domain 5 slightly enhanced Gemin5 interaction, and RNAs having either

an Sm-like or a scrambled sequence in the 5' end region exhibited similar binding capacity. Nevertheless, other IRES domains containing hairpins were not effective binding sites of Gemin5, indicating that not any stem-loop RNA structure provides a recognition motif for the protein.

As demonstrated by UV crosslinking and RNA-binding assays, the C-terminal region of Gemin5 is sufficient to bind directly to domain 5. The RNA-binding capacity of this region was unexpected, as no apparent similarity has been detected between the Gemin5 C-terminal region and known RNA-binding motifs. The C-terminal RNA-binding region differs from that described in earlier studies that identified the WD repeats domain as the region responsible for snRNA interaction (45). Differences in amino acid composition and, likely, in structural organization of these distant regions of Gemin5 may explain the diversity of RNA motifs recognized by the N-terminal, containing the 13WD repeats, or the C-terminal region of the protein.

Functional analysis involving expression of the protein in cell-free systems provided insights into the mechanism conferring its translation control capacity. The repressor effect of Gemin5 in mRNA translation, although moderate, is consistent with its capacity to compete for the RNA-binding site in the IRES element with PTB. Besides, incubation of Gemin5 and PTB with the FMDV IRES reorganized the local RNA structure of this region, resulting in a modified SHAPE reactivity of the region immediately upstream of the functional start codon. Gemin5 translational control may become significant in the context of a competitive environment, a

situation that is compromised during picornavirus infection. In this regard, we have recently reported that Gemin5 is proteolysed during FMDV infection (27). Gemin5 proteolysis occurred at similar times that PABP or PTB, and later than eIF4G cleavage, presumably contributing to inhibit cellular gene expression by eIF4G-independent mechanisms.

Gemin5 is found mainly in the cell cytoplasm (46) and a significant amount is not associated to other Gemins or SMN complex (25). Indeed, proteomic analysis of the SMN complex revealed that Gemin5 is a peripheral protein (47). Consistent with this finding, the SMN complex has been described as a dynamic system containing a large number of factors (48). The presence of free Gemin5 in the cell cytoplasm is congruent with a multi-functional role of this protein. Hence, it is likely that Gemin5 may recruit (or interfere with) other factors that also have mRNA-binding capacity and thus, regulate translation. Importantly, we have discovered that Gemin5 directly binds to a specific region of the IRES, and it is able to out-compete ITAFs that recognize the same region. Several alternative though not mutually exclusive possibilities may explain this property. One is the existence of similar recognition motifs in the competitor proteins despite no canonical RNA recognition motifs have been detected in the C-terminal region of Gemin5. The second one could be potential protein–protein interactions preventing the interaction of ITAFs with the IRES element. Following on this, interactions of Gemin5 with other RNA-binding proteins have been described, as illustrated by hnRNP U or eIF4E (20,49). hnRNP U is a RGG protein that interacts with the SMN protein (50) and coimmunoprecipitates with Gemin5 crosslinked to HCV and FMDV IRES (20). Conversely, proteins coimmunoprecipitating with hnRNP U included Gemin5 and eIF4E. Furthermore, M⁷GTP pull-down assays resulted in the isolation of eIF4E, Gemin5 and hnRNP U, but not PTB, irrespectively of the presence of viral IRES RNA (20), suggesting that interference with PTB is unrelated to protein–protein interaction. A third possibility could be that the RNA-binding sites of the competitor proteins are closely located leading to steric interference or to conformational changes in RNA structure. Our results of Gemin5–PTB interference, which promote a local RNA structure reorganization detected by the SHAPE nucleotide dynamics assay, favour the latter possibility. Our findings will be instrumental to determine the role of Gemin5, and Gemin5-associated partners, in translation initiation driven by other IRES elements, in particular, and translation control in general.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–5.

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